

Guanine and Inosine Nucleotides, Nucleosides and Oxypurines in Snail Muscles as Potential Biomarkers of Fluoride Toxicity*

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The aim of the present study was to determine the toxicity of fluorides on energy metabolism in muscles of the *Helix aspersa maxima* snail. Qualitative and quantitative analysis of purine compounds was performed in slices of foot from mature snails with high-performance liquid chromatography. Fluoride concentrations were measured using an ion-selective electrode and gas chromatography. The results show that exposure to fluoride pollution was accompanied by a statistically significant increase in fluoride concentrations in soft tissues. This effect was already noticeable with the smallest fluoride dose. Accumulation was greatest in the shell. There is a significant and positive correlation between fluoride concentrations in foot muscles and guanine and inosine nucleotides or uridine content. The content of low-energy guanylate, inosylate and oxypurine in foot muscles significantly increased with rising dose of fluoride. The difference as compared with controls was significant only for the highest dose of fluoride. Interestingly, uric acid, the final product of purine catabolism, dominated quantitatively in the foot muscles of snails. In conclusion, increased low-energy guanylate and inosylate as well as decreased xanthine concentrations in snail muscle can be indicators of the toxic influence of fluoride on the organism. The measuring of fluoride accumulation in the shell is the most suitable bioindicator of fluoride pollution in the environment.

Key words: Nucleotides, biomarker, fluoride, snail.

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Molluscs are generally recognized as convenient bioindicators of environmental contamination. The toxicity of fluorine to plants, animals and humans has been well documented (MILLER 1997). JĘDRZEJUK and MILEWICZ (1996) have shown that high doses of fluoride in food results in the inhibition of enzymes containing zinc, magnesium or manganese presumably because these metals are bound to and displaced by fluoride. Correlations between the content of fluoride in tissues of snails (*Helix pomatia*) have been reported. DWOJAK and MACHOY (2000) found correlations between the fluoride level in the environment and in snail shell. Some reports (MACHOY *et al.* 1995; ZAKRZEWSKA 1995) suggested that exposure of vertebrates to low levels of fluoride results in accumulation of fluoride associated with musculo-skeletal symptoms and metabolic disorders. RAĆ

(2005) observed that AMP (adenosine monophosphate) and AEC (adenylate energy charge) = $(ATP + ADP/2)/(ATP + ADP + AMP)$ measured in snail muscle may be used as biomarkers in environmental monitoring. The AMP or AEC value can be an estimate of the condition of the whole organism subjected to the toxic influence of fluoride.

The existing state of knowledge about transformations of purines in snails is presented in Figure 1 and based on RAĆ (2003).

The aim of the present study was to determine the toxicity of fluorides on the energy metabolism in muscles of the snail *Helix aspersa maxima*. The internal energy status of the cell can be estimated on the basis of measurements of concentrations of guanine and inosine nucleotides (GTP – guanosine triphosphate, GDP – guanosine diphosphate, GMP – guanosine monophosphate, IMP – inosine mono-

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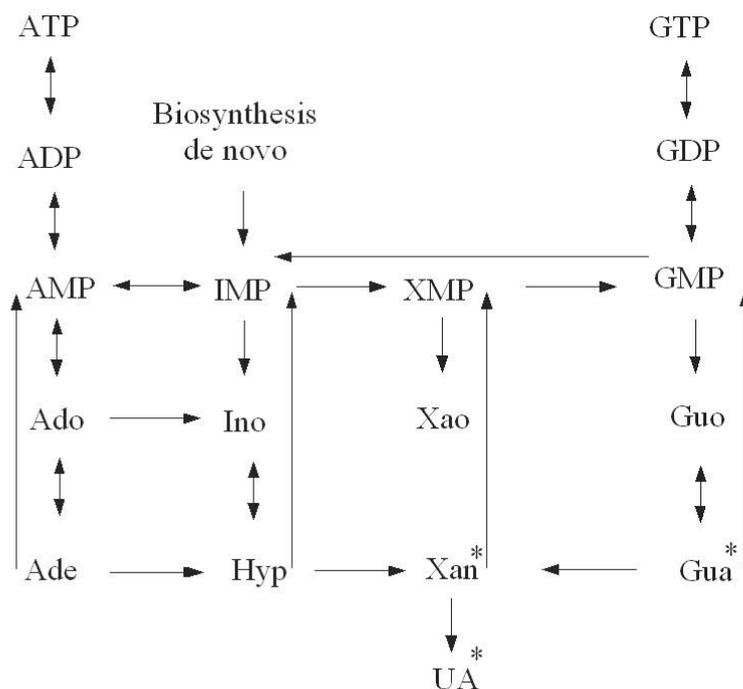


Fig. 1. Diagram of purine metabolism in hepatopancreas of snails (ATP – adenosine triphosphate, ADP – adenosine diphosphate, AMP – adenosine monophosphate, Ado – adenosine, Ade – adenine, IMP – inosine monophosphate, Ino – inosine, Hyp – hypoxanthine, XMP – xanthosine monophosphate, Xao – xanthosine, Xan – xanthine, UA – uric acid, GTP – guanosine triphosphate, GDP – guanosine diphosphate, GMP – guanosine monophosphate, Guo – guanosine, Gua – guanine). * – final products of purine catabolism in snails.

phosphate) and their metabolism degradation products (nucleosides Guo – guanosine and Ino – inosine), oxypurines (Hyp – hypoxanthine, Xan – xanthine, UA – uric acid) and one pyrimidine nucleoside: Urd – uridine with high-performance liquid chromatography (HPLC). We have chosen a species which is characterized by a high resistance to fluoride pollution (DWOJAK & MACHOY 2000) and low AEC (DWOJAK *et al.* 2000) so that fluoride effects on energy metabolism should be visible.

Material and Methods

The study was performed on *Helix aspersa maxima* snails removed from aestivation. Snails were given standard pulverized food (recommended by the Institute of Zootechnics in Balice) supplemented with varying doses of sodium fluoride (Table 1). A control culture without fluoride supplementation was run in parallel. Prior to collection of material, the snails were kept without food for 48 hours and then killed by freezing in liquid nitrogen. The experiment was started with 25 snails in each group. Only snails alive at the end of the experiment were used for chemical analysis (a variable number of the sample in Table 1), because a few snails died in each culture group during the experiment. Quantification of fluoride levels was done in soft tissues, foot and hepatopancreas, and also in shells of mature snails. Qualitative and

quantitative analyses of purine compounds were performed in slices of foot from mature snails.

Fluoride concentrations in pulverized shells were measured using an ion-selective electrode. Gas chromatography was used to determine fluoride concentrations in the hepatopancreas and foot. Purines were measured in foot muscle slices with high-performance liquid chromatography (HPLC).

Fluoride determinations using an ion-selective electrode were done according to the method of LASSOCIŃSKA *et al.* (1982) as modified for shells by the authors. Details of this method have been described by RAĆ *et al.* (2005). The fluoride con-

Table 1
Details of experimental protocol

Culture group with number of samples in 40th day of the experiment	Concentration of fluoride ions in food ($\text{mg}\cdot\text{kg}^{-1}$)	Culture duration
A ₁ = 10	control ^a	40 days
A ₂ = 23	133	
A ₃ = 14	665	
A ₄ = 22	1330	

^aconcentration in tap water was $0.15 \text{ mgF}^{-1}\cdot\text{l}^{-1}$

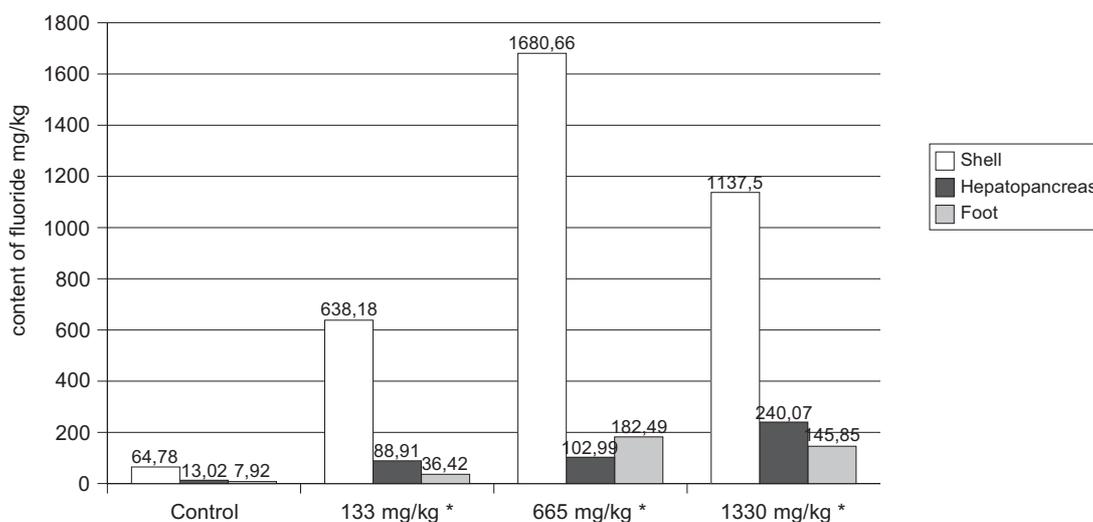


Fig. 2. Content of fluoride [mg kg^{-1} dry mass] in tissues of *Helix aspersa maxima*, *level of significance $P < 0.0001$ (Mann-Whitney U-test) as compared with controls (in each tissue).

Table 2

Median (interquartile range) of fluoride concentrations [$\text{mg} \cdot \text{kg}^{-1}$ dry mass] in tissues of *Helix aspersa maxima* exposed to rising concentrations of fluoride [mg kg^{-1} food]

Fluoride dose $\text{mg} \cdot \text{kg}^{-1}$	Shell	Hepatopancreas	Foot
Control	64.80 (44.19)	13.02 (7.44)	7.92 (1.76)
133	638.18* (484.2)	88.91* (101.85)	36.42* (10.50)
665	1680.66* (407.05)	102.99* (84.03)	182.49* (56.18)
1330	1137.50* (607.31)	240.07* (142.05)	145.85* (40.12)

*level of significance $P < 0.0001$ (Mann-Whitney U-test) as compared with controls.

centration was read with an OP 262 apparatus (Radelkis, Budapest, Hungary). Quality analysis was checked by measurement of NaF standard solutions as external standards. Fluoride content was calculated based on the weight of the pulverized sample.

Fluoride measurements using gas chromatography were done according to the method of MIKOŁAJEK *et al.* (1996) with some modifications to account for material specificity. The supernatant was withdrawn and transferred to a CHROM 5 (Laboratori Prístroje Praha) gas chromatograph equipped with a 2 m x 4 mm i.d. column filled with 20% modified silicone oil on Chromosorb P (DC200/50). Oven, injector and FID detector temperatures were 85°C, 220°C, and 270°C, respectively. Nitrogen served as a carrier gas flowing at 20 ml min^{-1} . Ali-

quots of 1 μl were injected every 20 min and the detector signal was sent to an IBM-PC class computer operating with dedicated software from Medson (Poznań, Poland). Concentrations of fluoride were adjusted for dry mass of sample taken for analysis. Details of this method have been described by RAĆ *et al.* (2005).

Determinations of purines with high-performance liquid chromatography were carried out according to the validated method of SAFRANOW *et al.* (2005), modified to account for material specificity. A small fragment was cut off from the foot at maximum contraction, with care to maintain consistency as to size and site of collection. The material was transferred to a 2 ml Eppendorf tube and immediately frozen in liquid

nitrogen. Samples were next stored at -80°C . Prior to analysis the material was placed in a steel mortar with the pestle cooled in liquid nitrogen. Tissues were transferred with a spatula cooled in liquid nitrogen to Eppendorf tubes filled with $500\ \mu\text{l}$ $0.4\ \text{M}$ HClO_4 at 0°C for protein denaturation, mixed and immediately frozen to -80°C . The material was thawed for 15 s prior to transfer to a rotating blade microhomogenizer. The homogenate was centrifuged at $14\ 000\ \times\ \text{g}$ and 3°C for 5 min. The supernatant was neutralized with approximately $100\ \mu\text{l}$ K_3PO_4 to a pH of 6-7, placed in a vortex mixer and left for 10 min at 0°C . The sediment was centrifuged as described above and the supernatant was kept at -80°C . The sediment after the first centrifugation was frozen and used to determine protein content according to BRADFORD (1976). Purines were separated using a Hewlett-Packard series 1050 liquid chromatograph equipped with a quaternary gradient pump with G1322A vacuum degassing module, desalting module, injection valve with a $20\ \mu\text{l}$ loop, UV-Vis detector, thermostatted column compartment (series 1100, G1316A type), and an analytical column $150\ \text{mm} \times 4.6\ \text{mm}$ i.d. containing $3\ \mu\text{m}$ Hypersil BDS with 12 nm pores (Alltech Associates Inc., USA). Details of this

method have been described by RAĆ *et al.* (2005). There are no certified reference materials for purine quantification, so identification and calibration were based on external standards. Quality control was based on a mix of 16 purines, which was chromatographed. The chromatograms were recorded and integrated using an IBM-PC computer with HPLC ChemStation software (Hewlett-Packard, USA). Concentrations were adjusted for protein content of sample.

Statistical analysis was done with Statistica 5.1PL software and non-parametric tests: the Kruskal-Wallis Test, the Mann-Whitney U-Test and the Spearman Rank Correlation Coefficient.

Results

Concentrations of fluoride in tissues of *Helix aspersa maxima* are presented in Table 2 and Figure 2. Concentrations of purines in the foot muscle of *Helix aspersa maxima* are shown in Table 3 and Figure 3. Correlations between purines and fluoride in foot muscle of *Helix aspersa maxima* for different fluoride doses in food are presented in Figures 4-6.

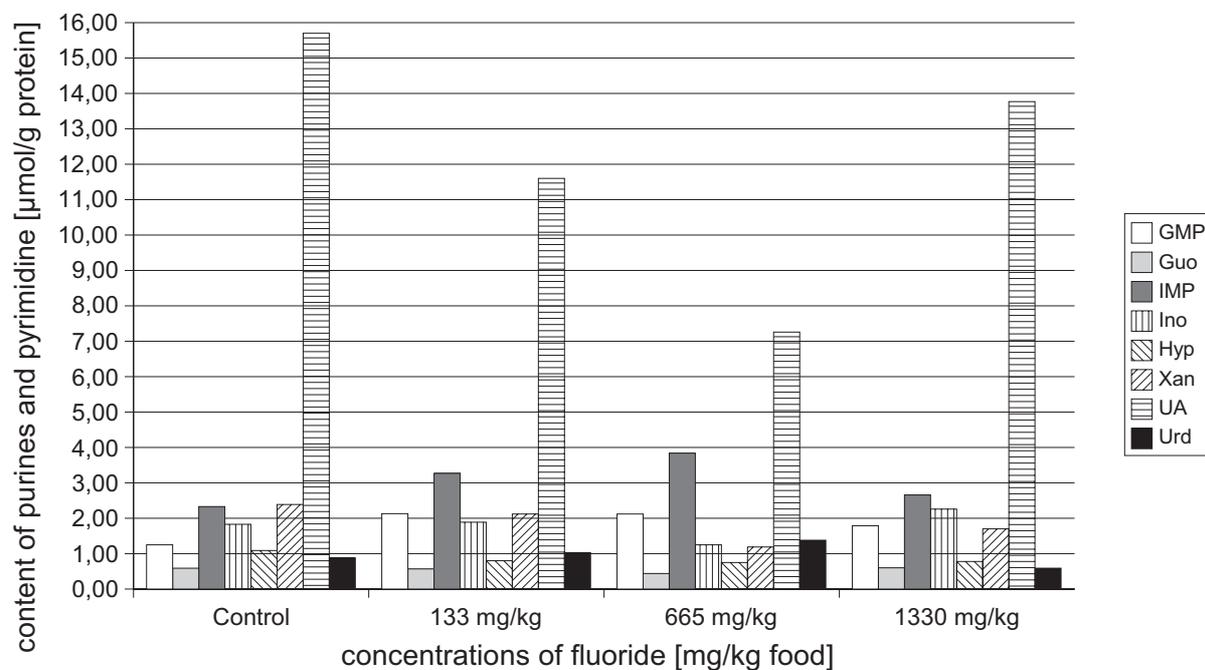


Fig. 3. GMP, Guo, IMP, Ino, Hyp, Xan, UA and Urd content [$\mu\text{mol g}^{-1}$ protein] in foot muscle of *Helix aspersa maxima* depending on fluoride dose in food (level of significance for differences between all groups $P < 0.05$ for GMP, Guo and Xan – Kruskal–Wallis ANOVA).

Table 3

Median (interquartile range) of purine content [$\mu\text{mol g}^{-1}$ protein] in foot muscles of *Helix aspersa maxima* exposed to rising concentrations of fluoride [mg kg^{-1} food]

Fluoride dose mg *kg^{-1}	GMP	Guo	IMP	Ino	Hyp	Xan	UA	Urd
Control	1.25 (1.18)	0.59 (0.25)	2.33 (1.33)	1.83 (1.13)	1.09 (1.07)	2.39 (2.1)	15.70 (22.63)	0.88 (0.97)
133	2.13* (1.35)	0.57 (0.36)	3.27 (4.13)	1.89 (1.42)	0.80 (0.67)	2.12 (1.25)	11.60 (11.02)	1.03 (1.69)
665	2.12* (0.76)	0.44** (0.26)	3.84** (2.31)	1.25 (0.67)	0.75 (0.33)	1.19* (0.38)	7.26 (5.66)	1.38 (1.66)
1330	1.79 (0.84)	0.60 (0.84)	2.66 (2.50)	2.26 (3.71)	0.78 (0.66)	1.70 (1.0)	13.77 (12.31)	0.59 (1.07)

*level of significance $P < 0.05$ (Mann-Whitney U-test) as compared with controls **level of significance $P < 0.01$ (Mann-Whitney U-test) as compared with controls.

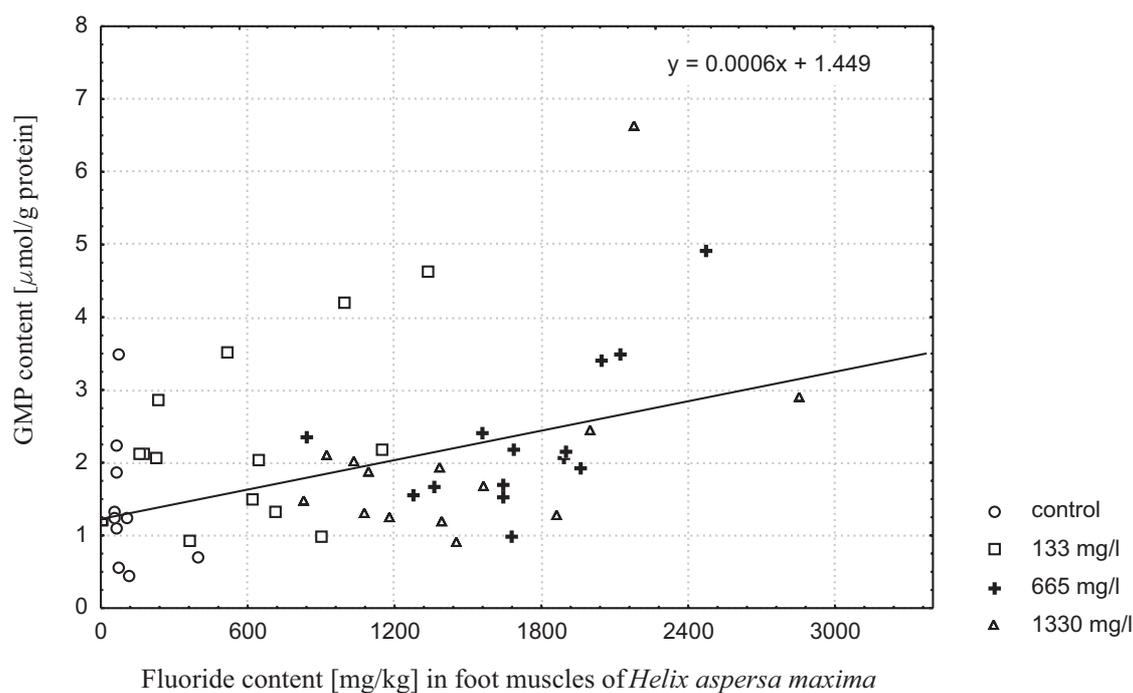


Fig. 4. GMP and fluoride content correlation in foot muscle of *Helix aspersa maxima* depending on fluoride dose in food (level of significance $P < 0.01$, Spearman rank correlation coefficient +0.34).

The results show that exposure to fluoride pollution was accompanied by a statistically significant increase in fluoride concentrations in soft tissues. This effect was already noticeable with the smallest fluoride dose. Accumulation was greatest in the shell (Fig. 2). None of the examined samples was found to contain detectable amounts of GTP and GDP (Table 3, Fig. 3). There is a statistically significant and positive correlation between fluoride

concentrations in foot muscles and GMP, IMP and Urd content (Figs 4-6). The content of low-energy guanylate (GMP) and IMP in foot muscles increased significantly with a rising dose of fluoride (Table 3, Figs 3-5). The difference in GMP concentrations as compared with controls was significant for the 133 and 665 mg/kg F⁻ doses. Statistically significant differences in IMP concentrations were noted only for the 665 mg/kg F⁻ dose (Table 3, Figs 3 & 5).

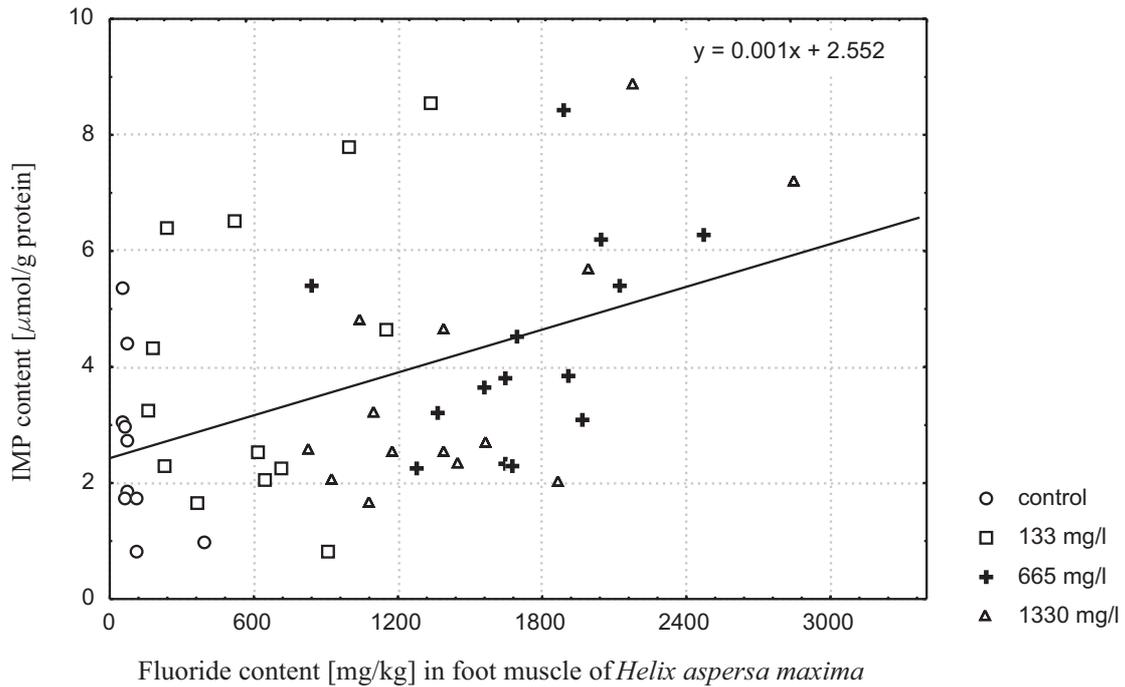


Fig. 5. IMP and fluoride content correlation in foot muscle of *Helix aspersa maxima* depending on fluoride dose in food (level of significance $P < 0.005$, Spearman rank correlation coefficient $+0.34$).

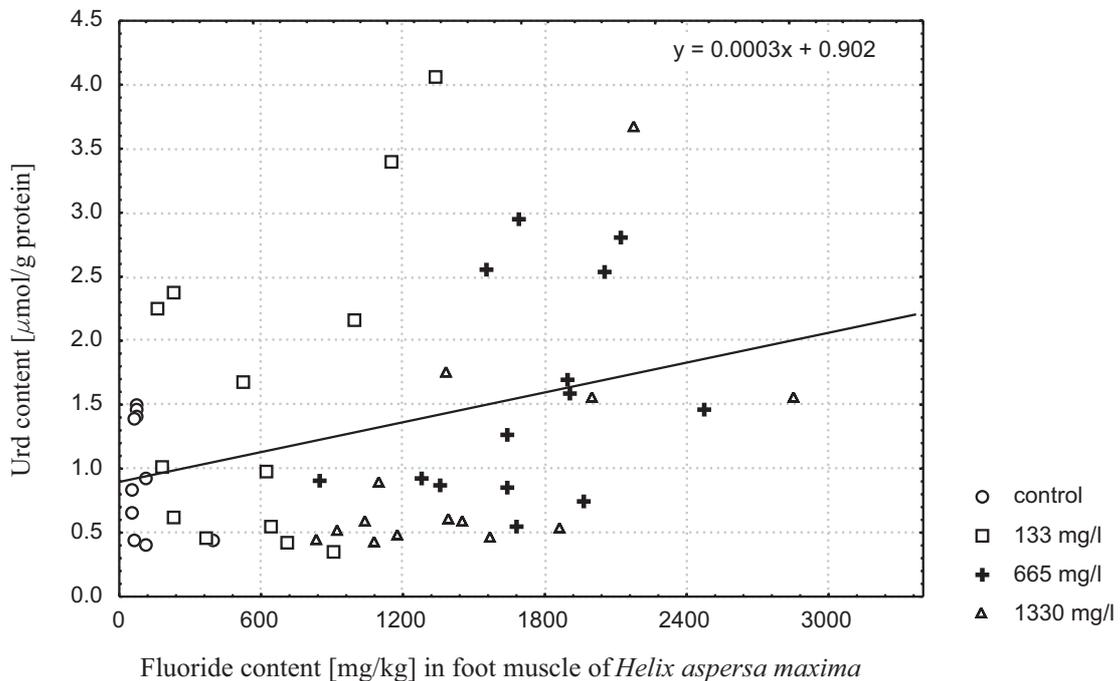


Fig. 6. Urd and fluoride content correlation in foot muscle of *Helix aspersa maxima* depending on fluoride dose in food (level of significance $P < 0.05$, Spearman rank correlation coefficient $+0.30$).

When the fluoride dose in food was 665 mg/kg, a decrease in concentrations of nucleoside (Guo) and oxypurine (Xan) in foot muscles were noted (Table 3, Fig. 3). Interestingly, UA, the final product

of the purine catabolism, dominated quantitatively in snail foot muscle (Table 3, Fig. 3). The content of Ino, Hyp and UA did not show statistically significant differences between groups (Table 3).

Discussion

Using enzymatic techniques other authors (BARANKIEWICZ *et al.* 1979 and JEŻEWSKA & SAWICKA 1968) determined the following substances in snail hepatopancreas: Gua, Ino, Xan, UA, AMP, IMP, GTP, ATP, ADP, Urd, Ctd (cytidine). Earlier reports (RAĆ *et al.* 2002) suggested that concentrations of purine indicators of the energy metabolism differ significantly in life and seasonal cycles. The most modern method of HPLC used in the present work identified the following purine derivatives in snail foot muscles, in the sequence of decreasing mean concentration: UA, IMP, Xan, Ino, GMP, Hyp, Urd, Guo. Despite using such a sensitive method, none of the examined samples was found to contain detectable amounts of GTP or GDP. The presented method could not resolve Gua, XMP and Xao.

Until now there are no publications about the influence of fluoride on the guanine and inosine nucleotides, nucleosides and oxypurines in snail muscles. In this work statistically significant differences in energy metabolism could be observed for the 665 mg/kg F⁻ dose, except for GMP levels in the foot which were elevated starting from the lowest concentration of fluoride used. None of the examined samples was found to contain detectable amounts of GTP or GDP, identified by other authors (DETERRE 1983). Similarly it was confirmed in previous studies (RAĆ 2003; RAĆ *et al.* 2005) that rising concentrations of fluoride lead to increased levels of AMP accompanied by an insignificant decrease in ATP and ADP levels. As a result, AEC was reduced in parallel to rising exposure to fluoride. Thus, a high fluoride concentration probably inhibits oxidative phosphorylation. Studies by DICKSON and GIESY (1982) have revealed that changes in AEC values in animal tissues are considered to reflect natural, as well as anthropogenic, transformations in the environment to a much greater extent than changes in concentrations of adenine nucleotides. Our earlier report (RAĆ *et al.* 2005) suggested that in cases of high levels of fluoride it seems reasonable to measure the content of AMP or the value of AEC which appear to serve as universal indicators of depressed metabolic function. In this work a positive correlation between fluoride concentrations in foot muscles and GMP seems to indicate that GMP measurement in snail muscle can be more suited as an estimate of the condition of the whole organism exposed to toxic fluoride.

VOGEL and OTTOW (1991) observed that tissue concentrations of fluoride in snails depend on the distance from the pollution emitter and that these molluscs are capable of achieving tissue levels between 207 and 808 µg g⁻¹ with maximum concen-

trations in the intestines and shell and minimum in reproductive organs. The results of this study indicate that there is significant accumulation of fluoride in soft tissues of snails. During chronic exposure to fluoride, the process of accumulation was most evident in the shell. Accumulation in the shell was significantly increased with the lowest concentration of fluoride with levels above those in the foot or hepatopancreas.

In conclusion, increased GMP and IMP as well as decreased Xan concentrations in snail muscle can be used as indicators of the toxic influence of fluoride on the condition of the whole organism. GMP measurement in snail muscle is the most suitable indicator of depressed metabolic function. Measurement of fluoride accumulation in the shell is the most suitable bioindicator of fluoride pollution in the environment.

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